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THE ROLE OF ALA134 IN CONTROLLING SUBSTRATE BINDING AND REACTIVITY IN ASCORBATE PEROXIDASE

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ABSTRACT

Ascorbate peroxidase (APX) is a class I heme peroxidase. It has two sites for binding of substrates. One is close to the γ -heme edge and is used for oxidation of ascorbate; the other is at the δ -heme edge and is used for binding of aromatic substrates [Gumiero *et al*, (2010) *Arch. Biochem. Biophys.* **500**, 13-20]. In this work, we have examined the structural factors that control binding at the δ -heme edge by replacement of Ala134 in APX with a proline residue that is more commonly found in other class II and III peroxidases. Kinetic data indicate that replacement of Ala134 by proline has only a small effect on the catalytic mechanism, or the oxidation of ascorbate or guaiacol. Chemical modification with phenylhydrazine indicates that heme accessibility close to the δ -heme edge is only minorly affected by the substitution. We conclude that the A134P mutation alone is not enough to substantially affect the reactivity of APX towards aromatic substrates bound at the δ -heme edge. The data are relevant to the recent application of APX (APEX) in cellular imaging.

INTRODUCTION

Heme peroxidases catalyse the H_2O_2 -dependent oxidation of a wide variety of substrates. The family of heme peroxidases share a common mechanism of oxidation which involves the formation of high-valent Compound I and Compound II intermediates [1]. These intermediates have been extensively studied in numerous peroxidase systems ever since the identification of these species in the 1950s [2, 3]. In contrast, it has taken much longer to understand the factors that control peroxidase substrate specificity. This was in part because the substrate specificity of peroxidase enzymes is very diverse but also because the actual physiological substrate is often difficult to identify. Most peroxidases are believed to oxidise small organic (usually phenolic) substrates under physiological conditions but there are prominent exceptions, notably cytochrome *c* peroxidase (CcP) and ascorbate peroxidase (APX) which use cytochrome *c* and ascorbate as their substrates, respectively. Many peroxidases will oxidise different kinds of substrate, probably at different locations [reviewed in [4]]. Initial predictions from chemical modification experiments [5-7] and from NMR (reviewed in [8, 9]) that substrates interacted with the heme at the δ -heme edge were later proved correct as crystal structures emerged for a number of substrate-bound complexes [4]. But the picture became more complex as a number of peroxidases – notably ascorbate peroxidase [10] and manganese peroxidase [11] – bind some substrates at the γ -heme edge instead.

In the case of ascorbate peroxidase (APX), we have established that there are two main sites for substrate oxidation. The first, close to the γ -heme edge, is used by ascorbate and is presumed to be the main physiological binding site [10, 12], Figure 1A. There is another binding site, close to the δ -heme edge [13], which is similar to that used in horseradish [14] and other [15, 16] peroxidases for closely related substrates. The δ -heme edge is widely regarded as the main site of oxidation for phenolic substrates (such as guaiacol) that are commonly used in peroxidase assays, but there is no direct structural evidence for binding of guaiacol at the δ -heme edge in any heme peroxidase and in CcP it is known that guaiacol binds at other locations [17]. The δ -heme edge is exposed to solvent in APX; Ala134 is also exposed to solvent and lies adjacent to the δ -meso position, Figure 1B (4.4 Å from δ -meso to C β of Ala134). The residue equivalent to Ala134 in APX is also present in CcP (Ala147) but is replaced with a proline in other class II and III peroxidases [18, 19], Figure 1C. To examine the role of Ala134 in controlling peroxidase reactivity at the δ -heme edge, we prepared a variant of APX in which Ala134 has been replaced with a proline and have examined the effect of this mutation on the

substrate specificity. This is relevant to the recent application of APX [20], and its A134P variant [21], in cellular imaging.

EXPERIMENTAL

Site-directed mutagenesis was performed according to the QuikChange™ protocol (Stratagene), using a wild-type pea cytosolic APX-encoding pMAL-c2 vector. Sequences for the forward and reverse oligonucleotides were 5'(CGCTTGCCTGATCCGACTAAGGGTTCTG)3' and 3'(GCGAACGGACTAGGCTGATTCCCAAGAC)5' respectively, with the 5' end phosphorylated and the bases underlined indicating the mutation site. Sequencing across the whole gene confirmed the presence of the required codon and the absence of other mutations. No alterations to published protocols [22] were required to successfully isolate A134P in moderate yields and with acceptably high purity ($R_z > 1.7$). An absorption coefficient of $\epsilon_{404} = 92 \text{ mM}^{-1}\text{cm}^{-1}$ for A134P, obtained using the pyridine hemochromagen method [23], was used to calculate enzyme concentrations.

Binding constants for binding of anionic ligands to the heme were determined according to previous protocols [24].

Steady state (pH 7.0, 25.0 °C) data were collected as previously reported [12] and were fitted either to the Michaelis-Menten equation or to the Hill equation ($v = V_{\max} [S]^n / (K^n + [S]^n)$), where v is the initial rate, n is a qualitative indication of the level of cooperativity, K is the substrate concentration at which the velocity is half-maximal, and V_{\max} is the maximum velocity. When $n = 1$, the Hill equation reduces to the more usual Michaelis-Menten equation ($v = V_{\max} / (1 + K_M/[S])$).

Pre-steady state (pH 7.0, 5.0 °C) kinetic experiments were carried out according to published protocols [24]. Compound I and Compound II spectra were obtained by addition of a 1:1 and 10:1 ratio of hydrogen peroxide to wild-type APX respectively. Second order rate constants for Compound I, k_1 , and Compound II, k_2 , formation were obtained as previously [24]; Compound II reduction was measured at 420 nm (Compound I/Compound II isosbestic point) after pre-formation of Compound I.

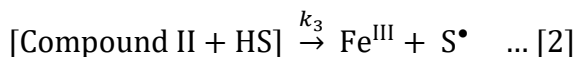
Phenylhydrazine modification was carried out by reacting the enzyme in the presence of the suicide substrate according to published protocols [22]; modification was complete within 1 minute in both cases. The extent of enzyme inactivation was measured by assaying with L-ascorbate, guaiacol and 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid according to published protocols [22]. Heme was extracted from inactivated enzyme as described previously and re-dissolved in a minimal volume (<100 μl) of the HPLC elution buffer. High performance liquid chromatographic analysis was carried out using a 5 μm Hypercil BDS C-18 column (4.6 mm x 250 mm, Thermoquest) with a computer-controlled

ProStar HPLC apparatus (Varian) and an isocratic acetonitrile/water/trifluoroacetic acid elution buffer (50:50:0.1 v/v).

RESULTS

Characterisation of A134P. The electronic absorption spectra of A134P has a Soret band that is slightly red-shifted ($\lambda_{\text{max}} = 404 \text{ nm}$) relative to the wild type ($\lambda_{\text{max}} = 403 \text{ nm}$ [22]). Wavelength maxima for various ligand-bound complexes of ferric A134P, Table 1, are similar to the equivalent species in the wild type enzyme. These spectra are consistent with the replacement of a residue that is remote from the heme iron which would not be expected to substantially perturb the properties of the heme group. The dissociation constants, K_D , for binding of cyanide ($K_{D,\text{cyanide}} = 0.6 \pm 0.1 \text{ } \mu\text{M}$) and azide ($K_{D,\text{azide}} = 235 \pm 59 \text{ } \mu\text{M}$) to the heme were also not significantly different from the wild type ($K_{D,\text{cyanide}} = 1.2 \pm 0.3 \text{ } \mu\text{M}$; $K_{D,\text{azide}} = 300 \pm 75 \text{ } \mu\text{M}$).

Pre-steady state kinetics. The Compound I intermediate of A134P forms normally (Figure 2, Table 1) and is consistent with a porphyrin π -cation radical formulation as has been established for the wild type protein [24]. Observed pseudo-first order rate constants ($k_{1,\text{obs}}$) for Compound I formation in A134P (data not shown) showed a linear dependence on H_2O_2 concentration in the range 0 - 15 μM H_2O_2 ($k_1 = 5.1 \pm 0.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ compared to $k_1 = 6.1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for wild type [24]). Wavelength maxima for Compound II of A134P, Figure 2, Table 1, are also in agreement with those for the wild enzyme [27]). Rate constants, $k_{2,\text{obs}}$, for reduction of Compound I to Compound II (data not shown) were linearly dependent on L-ascorbic acid concentration ($k_2 = 2.9 \pm 0.1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ compared to $k_2 = 2.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for wild type [24]). Reduction of Compound II showed saturation kinetics between 0 and 500 μM L-ascorbic acid (HS in equation [1] below), consistent with the following mechanism, equations [1] and [2]:



and an expression for $k_{3,\text{obs}}$ can be derived, equation [3] where [HS] is the concentration of ascorbate and K_d is the equilibrium dissociation constant for the substrate-bound complex ($K_d = 1/K_a$).

$$k_{3,\text{obs}} = \frac{k_3}{1 + K_d/[\text{HS}]} \quad \dots [3]$$

A non-linear fit of the data to equation [3], Figure 3, yielded $k_3 = 79 \pm 5 \text{ s}^{-1}$ and $K_d = 610 \pm 100 \text{ }\mu\text{M}$ for A134P ($k_3 = 73 \text{ s}^{-1}$ and $K_d = 560 \text{ }\mu\text{M}$ for wild type).

These data show that the rate constants in the peroxidase reaction mechanism are not significantly affected by the replacement of A134 by proline.

Steady state kinetics – oxidation of ascorbate and guaiacol. The steady-state oxidation of ascorbate by APX does not obey simple Michaelis-Menten kinetics, and a sigmoidal dependence of rate on substrate concentration is observed [25]. The A134P variant behaves similarly and plots of initial rate ($\mu\text{M s}^{-1}$) versus L-ascorbic acid concentration show a non-hyperbolic response, Figure 4. Steady state constants are given in Table 2. In contrast, oxidation of guaiacol by A134P exhibited conventional Michaelis-Menten type kinetics (data not shown); values for k_{cat} , K_M and k_{cat}/K_M , are given in Table 2 and are essentially as for the wild type protein.

Chemical modification – Phenylhydrazine modification of horseradish leads to the formation of both δ -mesophenyl and 8-hydroxymethyl heme derivatives [5, 6]. The method involves the formation of a phenyl radical through the oxidation of phenylhydrazine during peroxidase turnover with peroxide, and the product distributions have been interpreted as evidence that aromatic substrates interact at the exposed δ -position of the heme. Phenylhydrazine modification of APX has been shown [22] to produce the same δ -mesophenyl and 8-hydroxymethyl heme products, although higher proportions of δ -mesophenyl heme are observed compared to HRP. In Figure 5 are shown the HPLC traces of the heme-derived components extracted from phenylhydrazine-modified wild type APX and A134P. The data for A134P indicate that the product distribution is similar to that of wild type APX and is dominated by formation of the δ -mesophenyl heme (Figure 5). In HRP, which also contains a proline (Pro141) at the equivalent position, lower relative amounts of δ -mesophenyl heme are observed compared to the 8-hydroxymethyl product, but we do not interpret those differences as meaningful in terms of overall reactivity. The main points to note here are: (i) that both APX and HRP show the same modified products, consistent with substrate interactions at the δ -heme edge, and (ii) that the insertion of a proline does not substantially alter the product distribution in APX (see Discussion).

Attempts to decrease heme accessibility through introduction of a Phe residue at position 134 were unsuccessful because the A134F variant was isolated as the apo-form from *E. coli* and reconstitution protocols with exogenous heme did not generate catalytically active enzyme in our hands.

DISCUSSION

There is only one crystal structure for a peroxidase (CcP) in complex with guaiacol [17], and this shows that guaiacol is bound at two different locations, neither of which are close to the δ -meso edge. However, inhibition data and mutagenesis [17] strongly suggest that the catalytic binding site for aromatic compounds can occur the δ -heme edge in CcP. The observation that binding of guaiacol is relatively nonspecific (and of low affinity) is consistent with the widespread activity of all peroxidase enzymes for guaiacol. It is likely, therefore, that APX also contains multiple (non-specific) binding site for phenolic molecules in addition to a binding site close to the δ -heme edge.

We sought to probe the reactivity at this δ -heme edge, and to make comparisons with other peroxidases. Our data show that the oxidation of ascorbate is largely unaffected by the A134P mutation, which is consistent with ascorbate binding close to the γ -heme edge [10]. Oxidation of guaiacol was also not substantially altered in the A134P variant. The chemical modification experiments demonstrate that introduction of a proline in APX does not substantially change the product distribution compared to wild type [22]. Our conclusion is that the introduction of a proline in A134P does not affect accessibility of the δ -meso position of the heme and does not affect reactivity of the enzyme towards guaiacol. By comparison with CcP above, it is likely that guaiacol oxidation in the A134P variant occurs from a number of binding sites – this would explain the steady state data (which are unaffected by the mutation) and would be in agreement with previous structural work on CcP [26] which shows that access to the heme is hindered in the A147Y and A147M variants while guaiacol activity is not greatly affected.

These data are relevant to the recent application of APX [20], and its A134P variant [21], in cellular imaging using electron microscopy (EM). The EM method uses the intrinsic activity of APX towards aromatic substrates (diaminobenzidine in this case). The A134P variant of APX is observed [21] as a more sensitive reporter in cells than the wild type protein and this has been attributed to an increased stability of the A134P variant to peroxide-induced inactivation (although we note that inactivation by phenoxyl radical attack [27] might also be possible). We have not assessed the mechanisms of inactivation in this work, but our data demonstrate that the A134P mutation alone does not affect the reactivity of APX towards aromatic substrates bound at the δ -meso position and thus would not account for the enhanced sensitivity of A134P in cellular imaging. Further enhancement of EM contrast through substantially enhanced aromatic substrate oxidation by APX will thus require more than a single mutation.

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TABLE 1

Wavelength maxima (nm) for various derivatives of wild type APX and A134P (pH 7.0, 25.0 °C). ^{sh} denotes unresolved shoulder.

Derivative	Wild type	A134P
Fe ^{III}	403, 506, ~540 ^{sh} , 636	404, 500, ~540 ^{sh} , 636
Fe ^{III} -CN ⁻	419, 540, 572 ^{sh}	420, 540, 572 ^{sh}
Fe ^{III} -N ₃ ⁻	412, ~525, ~543, 569 ^{sh} , 633	413, ~525, ~543, 569 ^{sh} , 633
Compound I	404, 529, 583 ^{sh} , 650	406, 527, 584 ^{sh} , 651
Compound II	414, 528, 559	414, 530, 557

TABLE 2

Steady state kinetic parameters^a (k_{cat} , K_M) and selectivity coefficients (k_{cat}/K_M) for the oxidation of L-ascorbic acid and guaiacol by rpAPX and A134P.

	L-Ascorbic acid ^b			Guaiacol		
	k_{cat}	K_M	k_{cat}/K_M	k_{cat}	K_M	k_{cat}/K_M
	(s ⁻¹)	(μM)	(μM ⁻¹ s ⁻¹)	(s ⁻¹)	(mM)	(mM ⁻¹ s ⁻¹)
Wild type	248 ± 28	410 ± 40	0.61	66 ± 3 ^b	12.3 ± 0.9	5.4
A134P	264 ± 44	440 ± 40	0.60	91 ± 6	16.4 ± 0.7	5.6

^a All data were fitted using the Michaelis-Menten equation, except ^b where data were fitted using the Hill equation.

^b Data from reference [24].

FIGURE LEGENDS

Figure 1. (A) Left: The structure of the APX/ascorbate complex, showing the ascorbate bound through the heme 6-propionate and Arg172, and the location of Ala134 close to the δ -meso site. The δ - and γ -heme locations are indicated. Right: A slightly rotated view, showing only the ascorbate and the position of Ala134 in relation to the overall protein structure. **(B)** The protein surface in APX. Left: Viewed looking towards the δ -heme edge, showing the heme group (in white) exposed to solvent; the δ -meso carbon of the heme and A134 are highlighted in red. Right: Viewed looking towards the γ -heme edge, showing the heme 6-propionate (in red) exposed to solvent; ascorbate binds on the surface at this site, as shown in **(A)**. **(C)** An alignment of the APX and HRP structures in the region of the δ -heme edge. The residues in HRP (Phe142, Pro141, Phe175) are labelled and are shown in pink; the corresponding APX residues (Asp133, Ala134, Thr135 and Phe175) are overlaid in green, with Ala134 indicated with a *.

Figure 2. UV-visible absorption spectra of ferric A134P (solid line) and its oxidized Compound I (dotted line) and Compound II (dashed line) intermediates in the Soret (top) and visible (bottom, multiplied by a factor of 5) regions. Conditions: 2 μ M APX, 5.0 $^{\circ}$ C, 0.1 M sodium phosphate pH 7.0.

Figure 3. Pseudo-first-order rate constants, $k_{3,obs}$, as a function of concentration of [L-ascorbic acid] for reduction of Compound II of wild-type APX (●) and A134P (○). The solid lines in both cases are a fit of the data to equation [3]. Conditions: [APX] = 0.5 μ M, 5.0 $^{\circ}$ C, 0.1 M sodium phosphate pH 7.0.

Figure 4. Steady-state oxidation of L-ascorbic acid by wild type (●) and A134P (○). Data were fitted to the Hill equation (see methods). Conditions: [APX] = 25 nM, [H₂O₂] = 0.1 mM, 25.0 $^{\circ}$ C, 0.1 M sodium phosphate pH 7.0.

Figure 5. Reverse-phase HPLC analyses of products derived from phenylhydrazine modification of wild type and A134P, monitored at 400 nm. Peaks corresponding to 8-hydroxymethyl heme, unmodified heme and δ -mesophenyl heme are indicated. Peaks were assigned according to previously [22].

Figure 1

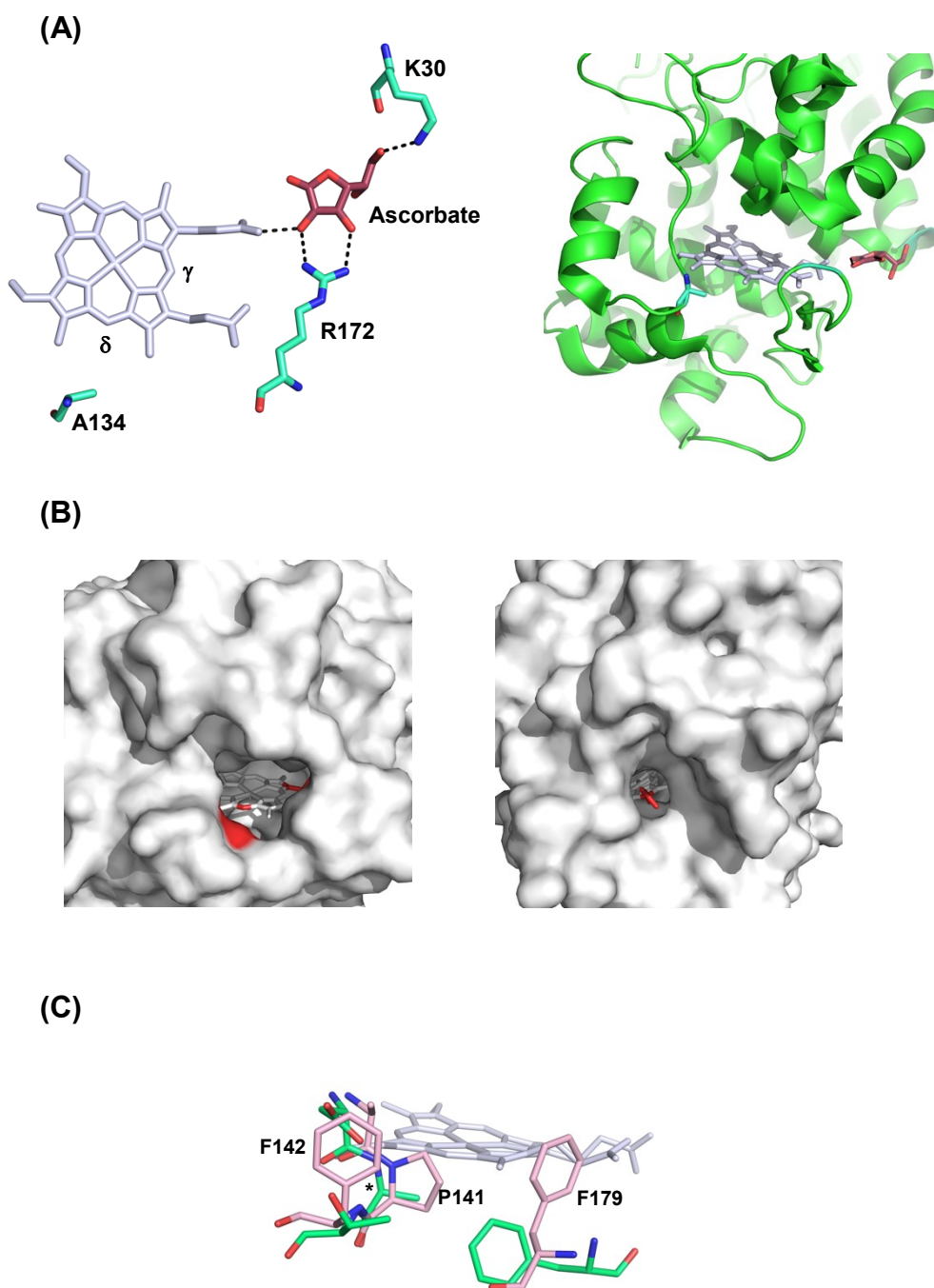


Figure 2

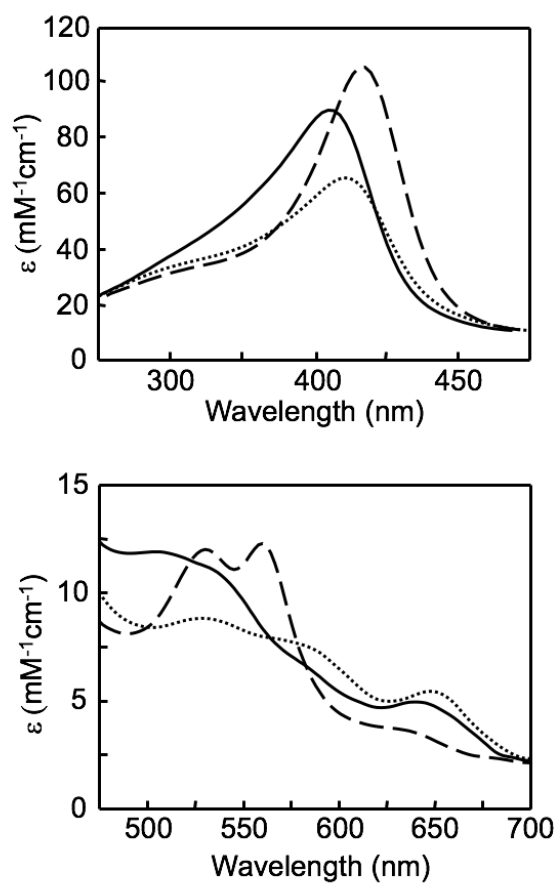


Figure 3

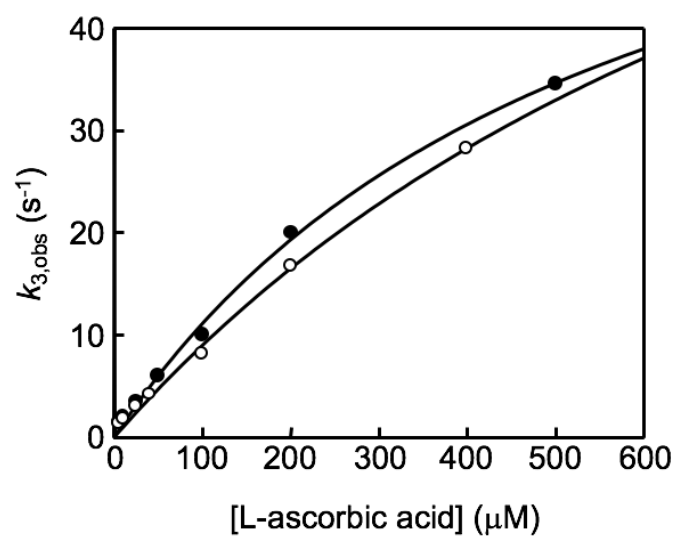


Figure 4

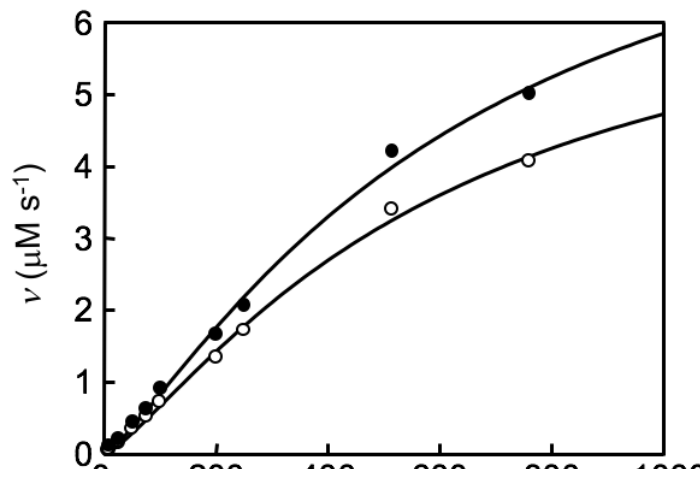
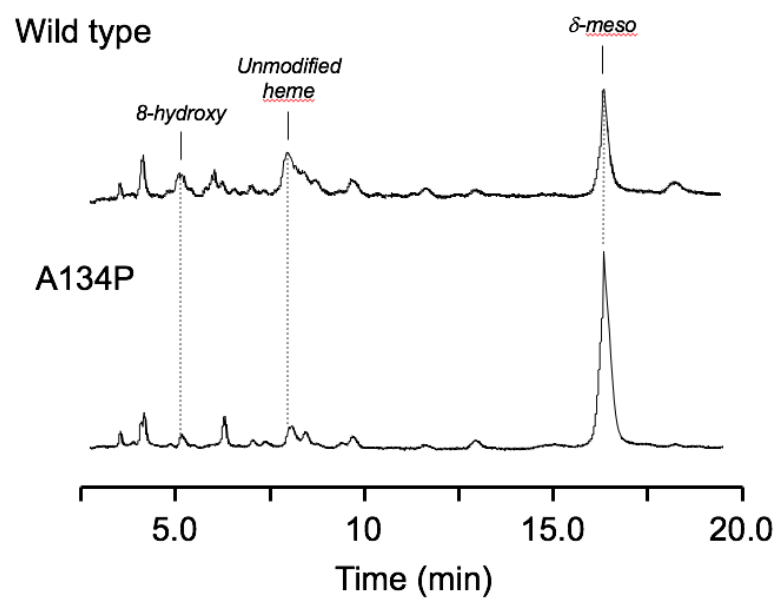


Figure 5



Synopsis

The role of Ala134 in controlling substrate specificity in ascorbate peroxidase was explored. Replacement of Ala134 by proline has a small effect on oxidation of ascorbate or guaiacol. We conclude that the A134P mutation alone does not substantially affect the reactivity of towards aromatic substrates bound at the δ -heme edge.

